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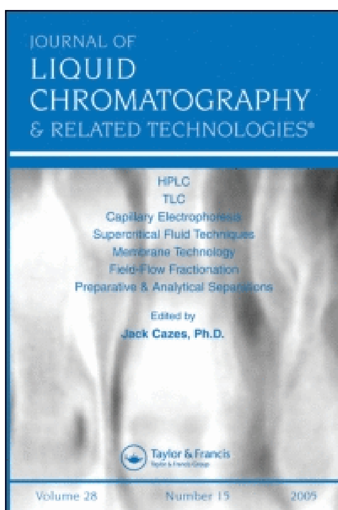

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Flow Cell CCC/FT-IR Spectrometry

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FLOW CELL CCC/FT-IR SPECTROMETRY

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ABSTRACT

The development of flow cell LC/FT-IR spectrometry has been plagued by the fact that liquid chromatographic mobile phases absorb infrared radiation. This problem is exacerbated by low solute-to-solvent ratios in the eluates. High speed countercurrent chromatography (CCC) is used in this study to obtain high solute-to-solvent ratios that alleviate some of the problems of infrared absorption of the mobile phase. The high solute-to-solvent ratios allow the use of a simple flow cell interface which makes complex solvent removal procedures unnecessary. CCC/FT-IR (Countercurrent chromatography/Fourier transform infrared) spectrometry is useful for applications where the analyst is not limited by sample size, and high sample loadings are possible. CCC/FT-IR spectrometry is particularly useful to users of countercurrent chromatography to obtain useful structural information of mixture components with an FT-IR spectrometer.

INTRODUCTION

Despite the fact that many systems for LC/FT-IR spectrometry have been designed and studied, FT-IR spectrometers are not widely used in conjunction

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with liquid chromatography. On the other hand FT-IR spectrometry continues to grow as a technique for gas chromatography (1). The large difference between the two hyphenated techniques is the fact that liquid chromatographic mobile phases are not infrared transparent, as is the case with gas chromatographic mobile phases. Solvents that serve as good mobile phases for liquid chromatography, are usually strong absorbers in the infrared region of the electromagnetic spectrum.

Another obstacle in the development of LC/FT-IR spectrometry has been the low concentrations of liquid chromatographic eluates. As most compounds elute from a normal phase HPLC column have a maximum concentration of approximately 20 ppm (2). If the effluent from a liquid chromatographic column is passed through an IR flow cell most of the absorption of infrared radiation is due to the mobile phase at these low concentrations. Since the mobile phase is present in much higher concentrations than the eluate, detection of the eluate is difficult or impossible because the absorbance of the eluate is in the noise of the solvent spectrum. FT-IR spectrometric detection systems have insufficient dynamic range to detect very small absorbances in the presence of very strong absorbances. Reversed phase HPLC, where the mobile phase is always strongly absorbing, is an excellent example of this situation.

Early work in LC/FT-IR spectrometry involved the use of chlorinated solvents as mobile phases and flow cells of short pathlengths. Chlorinated solvents such as chloroform and carbon tetrachloride have wide infrared spectral windows. Normal phase HPLC and size exclusion chromatography (SEC) separations have been carried out with these solvents (3-6). Short

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pathlength flow cells (<200 μm) were used in these studies to minimize the solvent absorbance. Even at these short pathlengths solvent opacity problems occurred and structural information from spectra of the eluates could not be recorded in areas of solvent opacity. The use of short pathlengths lowered the sensitivity of the system, since the absorbance due to the eluate was decreased along with that of the mobile phase. Working with flow cells of 0.025 and 0.20 mm pathlengths Teramae obtained spectra of about 20 μg of sample using carbon tetrachloride as the mobile phase (3).

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Every LC/FT-IR system is designed to overcome the problems presented by the infrared absorbing mobile phase. One approach to this problem is the removal of the mobile phase prior to infrared analysis. The "buffer memory" system developed by Jinno is an example of solvent removal LC/FT-IR spectrometry (7-9). Several research groups have developed solvent removal interfaces for both normal and reverse phase HPLC, in conjunction with diffuse reflectance spectrometry (10-15). The first system was developed by Griffiths and co-workers for normal phase HPLC (10,11). The same group has also developed a solvent removal system for reverse phase HPLC based on the methylene chloride extraction of Karger *et. al.*, that was first reported for LC/MS (12,16). Excellent detection limits, but slightly higher than those of their normal phase interface were reported for this system. In the solvent removal interface developed by Kalasinsky for reversed phase HPLC, water is eliminated by employing 2,2'-dimethoxypropane to convert water to methanol and acetone which are volatile products, and do not harm the KCl matrix (13). The liquid chromatographic eluent is deposited onto a KCl substrate that is held on a compartmentalized train or a continuous "trough" (14). In another

reversed phase HPLC/FT-IR spectrometry interface, Castles *et. al.* used diamond grit powder as their matrix for diffuse reflectance spectrometry (15). The mobile phase which was composed of methanol and water was removed with a piezoelectric nebulizer.

Taylor and coworkers have developed a flow cell microbore HPLC/FT-IR system for both normal and reversed phase HPLC (17-19). The main advantage of this system is it employs very little solvent. Solvents, such as $CDCl_3$ allow the analyst to obtain information about the C-H stretch region of the spectrum. These solvents can be used despite their high cost as only small quantities are used. Taylor has stated that concentrations up to 20 times those of conventional HPLC can be obtained with microbore HPLC. This is only true if the same concentration and quantity of sample is injected in both microbore and a conventional HPLC column. Peak volumes are lower with microbore HPLC but the sample loading capacity of the column is reduced in the same manner.

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More recently Hellgeth and Taylor have developed a post column extraction of solutes from reverse phase HPLC effluent so that a flow cell can be used (20). Griffiths has developed a warm nitrogen solvent evaporation device to remove the mobile phase and then deposit the chromatographic components on a substrate for spectral examination with an infrared microscope (21). Gagel and Biemann have developed a similar device (22).

In a previous study from this laboratory it was shown that high eluent concentrations can be obtained by using high speed countercurrent chromatography (CCC) (23, 24). High speed countercurrent chromatography permits high sample loadings without loss of chromatographic resolution (25-27). The high

speed CCC can operate at very high sample loadings, since it is a liquid-liquid chromatograph without a solid support for the stationary phase. These high solute-to-solvent ratios alleviate some of the problems of infrared absorption by the mobile phase, making complex solvent removal procedures unnecessary. A much simpler flow cell interface is used instead for CCC/FT-IR spectrometry. The system is ideal for applications where the analyst is not limited by sample size, and high sample loadings are possible.

MATERIALS AND METHODS

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Chromatography. The countercurrent chromatograph used has been extensively described elsewhere in the literature (25-27) and a review will not be given here. The chromatograph column spool, used in this study, was adapted to increase the centrifugal field applied to the column. These changes are described elsewhere (24). The high speed CCC used a 130 meter column with an inner diameter of 1.2 mm and a total capacity of 160 mL. This multilayer column has 5 layers of PTFE (polytetrafluoroethylene) tubing.

Two example separations are presented. The first was a two phase solvent system of chloroform, water (acidified to pH 2), and methanol in a ratio of 3:3:1. The two phases were equilibrated before being introduced into the column. This solvent system was used to separate acetophenone, benzoic acid, *p*-nitrophenol, and phenol. A total of 1100 theoretical plates was calculated for this separation. Partition coefficients and concentrations of the components, as well as other data, are summarized in Table I for this separation. The second separation was achieved using a 3:3:2 solvent system of hexane, methanol, and water. These two phases were also equilibrated before

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TABLE I.

Chromatographic Parameters for Chloroform:Water:Methanol System

Component	t_R[a] (min)	w_b[b] (min)	R_S[c]	K[d]	k'[e]	Amount Injected (mg)	Average Conc. (mg/mL)
Acetophenone	12.1	1.2		0.002	0.009	1.0	0.42
Benzoic Acid	18.9	2.4	3.8	0.13	0.59	1.3	0.27
p-Nitrophenol	23.1	3.6	1.4	0.21	0.96	1.2	0.17
Phenol	30.7	4.4	1.9	0.35	1.6	1.1	0.13

[a] Retention time in minutes.
[b] Peak width in minutes.
[c] Resolution = $2(t_{R_2}-t_{R_1})/(w_{b_1}+w_{b_2})$

[d] Partition coefficient = (Concentration in stationary phase)/(Concentration in mobile phase)
[e] Capacity factor = K(Volume of stationary phase)/(Volume of mobile phase)

being introduced into the column. This system separated benz[α]anthracene-7,12-dione, benzophenone, and *o*-nitrophenol. In this separation a total of 340 theoretical plates was achieved, and the parameters associated with this separation are given in Table II.

The aqueous phase was used as the stationary phase for both separations. After the column was filled with the stationary phase, a revolutional speed of 1,000 rpm was applied to the column and the mobile phase was introduced. Initially about 20% of the stationary phase was displaced before the elution of the mobile phase. From this point on, the mobile phase only displaced itself and no more stationary phase was lost. In the chloroform separation the mobile phase was pumped from the inner (head) end of the column, and in the hexane separation from the outer (tail) end. A flow rate of 2.0 mL/min was used for both separations.

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Spectrometry. The CCC/FT-IR spectra were obtained with a Digilab FTS-20/E/D spectrometer (Digilab Division of Bio-Rad, Cambridge, MA). A Mercury Cadmium Telluride detector (Infrared Associates, New Brunswick, NJ) was used. Spectra were measured at 8 cm^{-1} resolution, and no smoothing was performed on the spectra. The number of signal averaged scans varied; 12 or 15 scans were coadded. A commercially available micro demountable flow-through cell (Spectra-Tech, Inc., Stamford, CT) was used. CaF_2 windows were employed. Teflon tubing with a 0.3 mm i.d. was connected to the flow cell outlet to prevent the formation of gas bubbles. Pathlengths ranging from 25 μm to 1.0 mm were used in this study. The micro flow cell was used with a 4X reflecting beam condenser (Model 620 Spectra-Tech, Inc., Stamford, CT).

Reagents. Acetophenone and benzophenone were obtained from Fisher Scientific Co., (Fairlawn, NJ) phenol from Baker and Adamson, (Morristown,

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TABLE II.

Chromatographic Parameters for Hexane:Methanol:Water System

Component	t_R* (min)	w_b* (min)	R_S*	K*	k'*	Amount Injected (mg)	Average Conc. (mg/mL)
Benz[a]anthra-cene-7,12-dione	12.9	2.8		0.036	0.18	0.83	0.15
Benzophenone	21.5	4.8	2.3	0.20	0.98	2.5	0.26
o-Nitrophenol	36.1	7.6	2.4	0.47	2.3	2.5	0.16

* These terms are defined in Table I.

NJ) benzoic acid from Matheson, Coleman & Bell, Norwood, Ohio, and *o*-nitrophenol, *p*-nitrophenol, and benz[α]anthracene-7,12- dione from Eastman Kodak Co., (Rochester, NY). Doubly distilled water was used. Chloroform, hexane, and methanol, HPLC grade, were obtained from the J. T. Baker Chemical Company.

RESULTS AND DISCUSSION

In the CCC/FT-IR spectrometry flow cell studies presented here, pathlengths ranged from 0.025 mm to 1.0 mm. The regions of solvent opacity were minimized by using the 0.025 mm pathlength, but this also reduced the eluate absorbances. Figure 1 shows spectra of the compounds separated by the first system [3:3:1 chloroform, water (acidified to pH 2), methanol solvent system] when a 25 μm pathlength cell was used. The signal-to-noise ratios (SNR) obtained for these spectra demonstrate that the detection limit has not yet been reached. Two small areas of solvent opacity can be observed; around 3000 cm^{-1} and 1200 cm^{-1}. Below 800 cm^{-1} no spectral information is obtained due to the chloroform C-Cl stretch band and the MCT detector cutoff. The 1250 cm^{-1} acetophenone band can be observed even though chloroform, the main constituent of the chromatographic mobile phase, has a very strong absorbance band at 1200 cm^{-1}.

In flow cell LC/FT-IR spectrometry it is important to average as many scans as possible without averaging scans of lower SNR obtained before or after the peak maximum. The peaks from the 3:3:1 chloroform:water:methanol separation yielded 24 to 30 spectra of very high SNR around the peak

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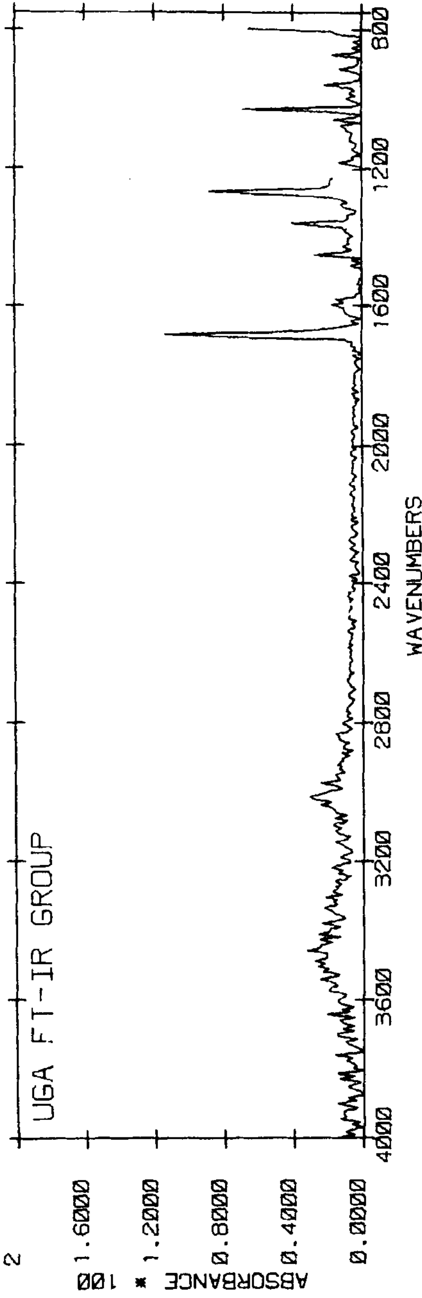

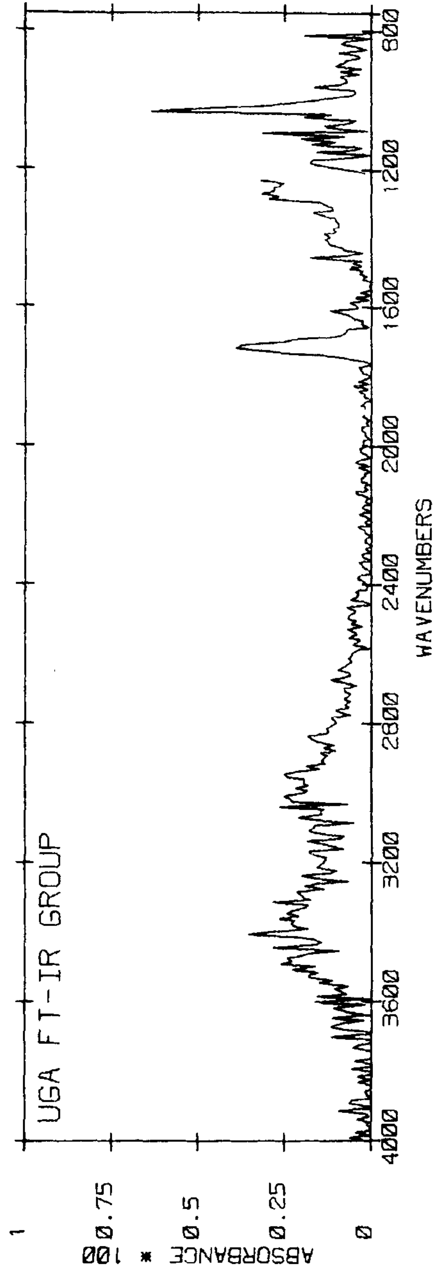

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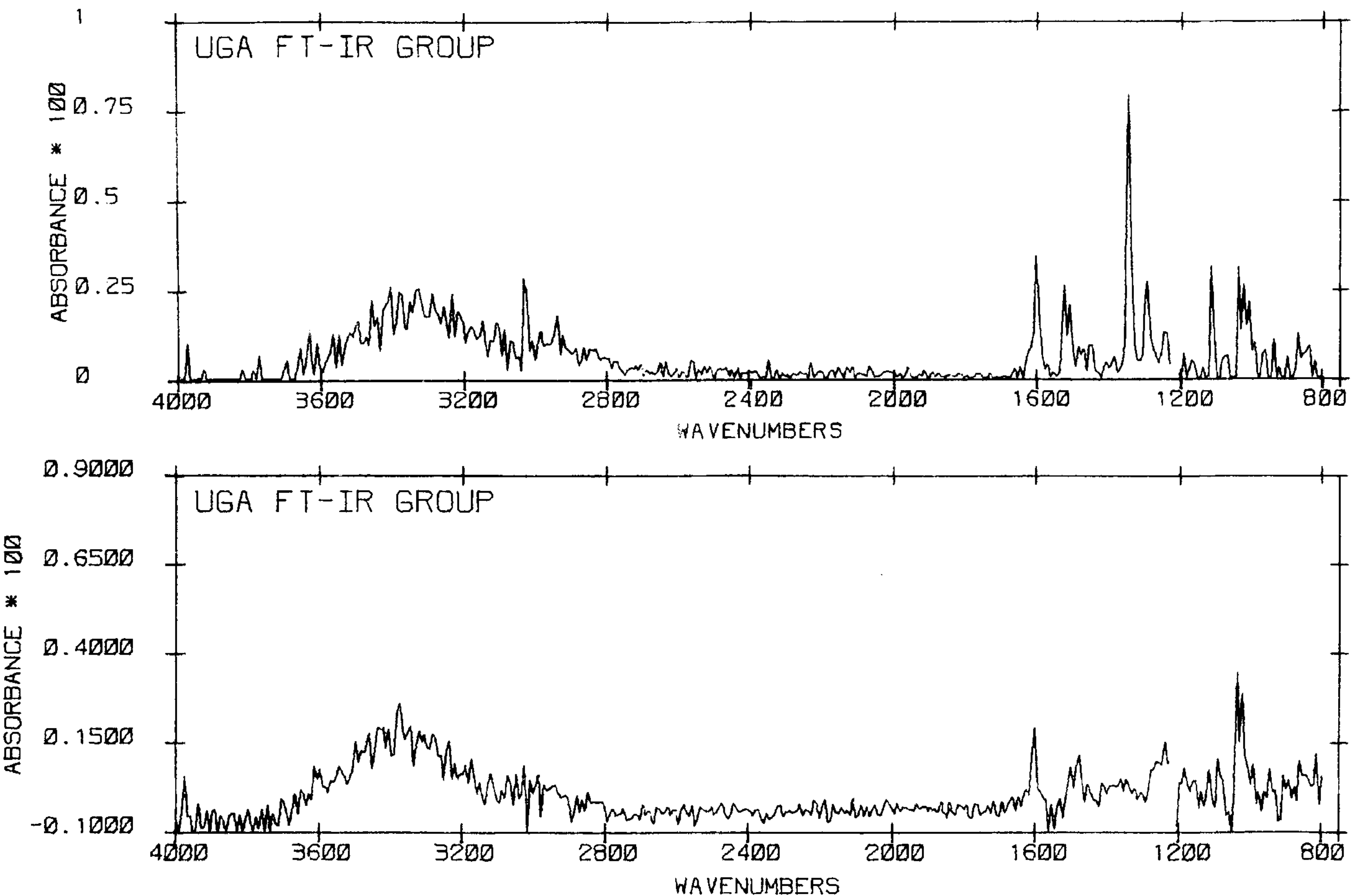

1. Spectra of a) 1.1 mg of acetophenone, b) 1.9 mg of benzoic acid, c) 1.3 mg of *p*-nitrophenol, and d) 2.0 mg of phenol, at a pathlength of 25 μm.

maximum. The spectra presented here were obtained by averaging twelve or fifteen scans to insure that at least one spectrum of the peak maximum was obtained. This sampling criterion greatly differs from the one employed for GC/FT-IR spectrometry, where the volume of the light pipe is chosen to equal or be slightly less than the width at half height of the narrowest peak in the separation (28). As gas chromatographic mobile phases do not absorb infrared radiation, in GC/FT-IR spectrometry the fraction of the peak within the light pipe is maximized. The same criterion cannot be applied to LC/FT-IR spectrometry where the mobile phase absorbs infrared radiation. Instead the volume of the flow cell should be small enough that it will capture the most concentrated part of the peak.

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Water has always been a difficult problem in LC/FT-IR spectrometry even with solvent removal systems (12-15). Methylene chloride has been used to extract the eluents in reversed phase HPLC/FT-IR spectrometry (12) but water still interfered as water is slightly soluble in methylene chloride. These problems might also be expected from the system described in this paper. As discussed in the previous section, the solvent system was formed by equilibrating chloroform, water (acidified to pH 2), and methanol in a ratio of 3:3:1. The upper aqueous layer was used as the stationary phase and the lower organic layer as the mobile phase. Since methanol is miscible with chloroform and water, and water is also slightly soluble in chloroform, problems such as those encountered by Griffiths might be expected. Nevertheless, the spectra shown in Figure 1 (a-d) show that neither water nor methanol presented serious problems, even though some evidence of water can be seen in the broad band at 3500 cm^{-1}. Since the concentrations of water and methanol in

the mobile phase are low compared to that of the eluent they can be easily removed by ratioing without affecting the quality of the spectra.

Spectra from the second separation (3:3:2 hexane, methanol, water solvent system) are shown in Figure 2 (a-c). The most striking feature of these spectra is the effect produced by the hexane C-H stretching band. This band totally obscures the region from 3000 to 2800 cm^{-1}. The region from 1500 to about 1350 cm^{-1} is also opaque due to strong hexane bands. These bands obliterate much of the *o*-nitrophenol spectrum. In the region between 1350 and 800 cm^{-1}, however valuable structural information can be obtained. In this region more information can be obtained with hexane than with chloroform. In some of these spectra small bands around 1640 cm^{-1} indicate the presence of water; but at a small enough concentration that it does not cause any problems.

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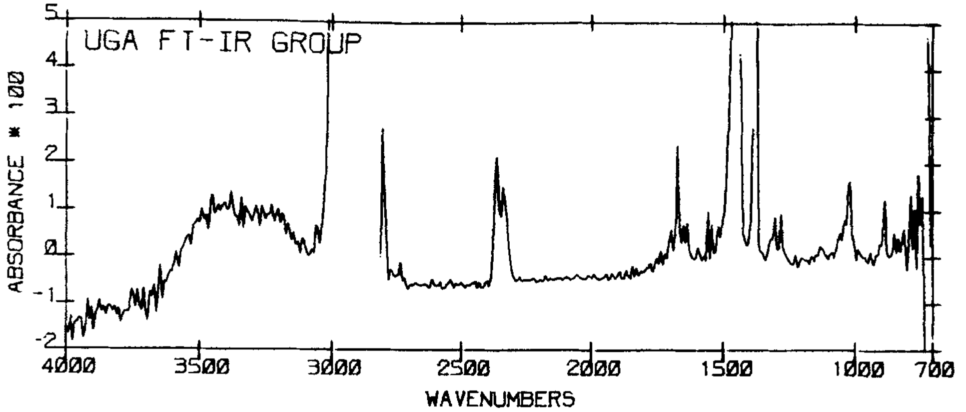
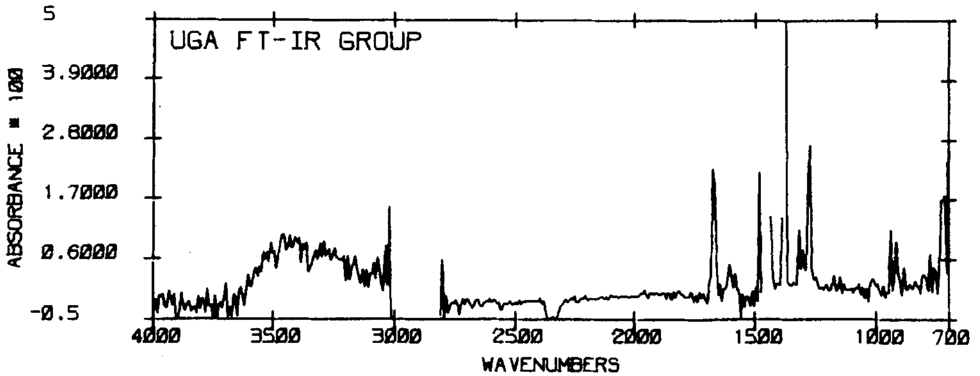
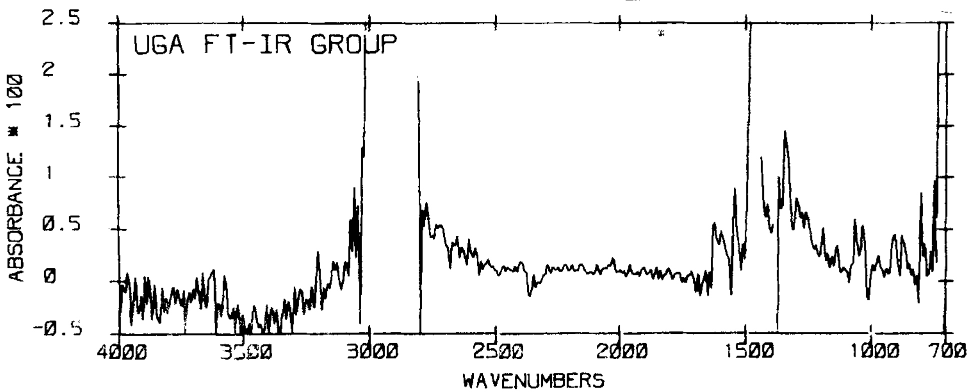
A pathlength of 0.5 mm was also used for the detection of components separated by the 3:3:1 chloroform, water, methanol solvent system. At higher pathlengths spectra of lower amounts of the components of interest can be obtained. The absorbance due to the solvent is also increased, and the number of areas of solvent opacity might be augmented (see Figure 3). A new area of solvent opacity now exists from about 3600 to 3700 cm^{-1}. The regions of opacity observed at 0.2 mm pathlength were also slightly expanded.

As previously mentioned, CCC/FT-IR spectrometry was developed for cases where the analyst is not limited by sample size, and high sample loadings can be used. All of the spectra presented up to this point were obtained when more than 1 mg of each compound was injected. By using a longer pathlength (0.5 mm), spectra were obtained for quantities ranging from 200 to 270 μg for







2. Spectra of a) 830 μg of benz[α]anthracene-7,12,dione, b) 2.5 mg of benzophenone, and c) 2.5 mg of *o*-nitrophenol, at a pathlength of 0.2 mm.

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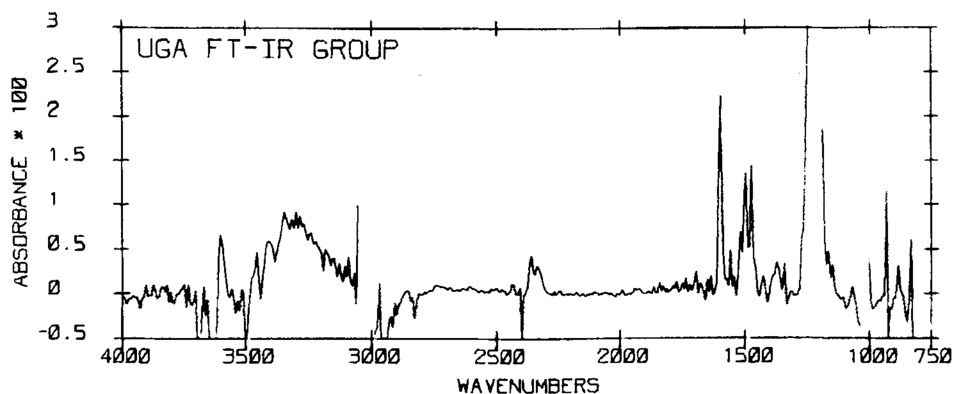

3. Spectrum of 1.1 mg of phenol at a pathlength of 0.5 mm. The longer pathlength offers greater sensitivity, but solvent opacity problems are greater (compare to Figure 1d).

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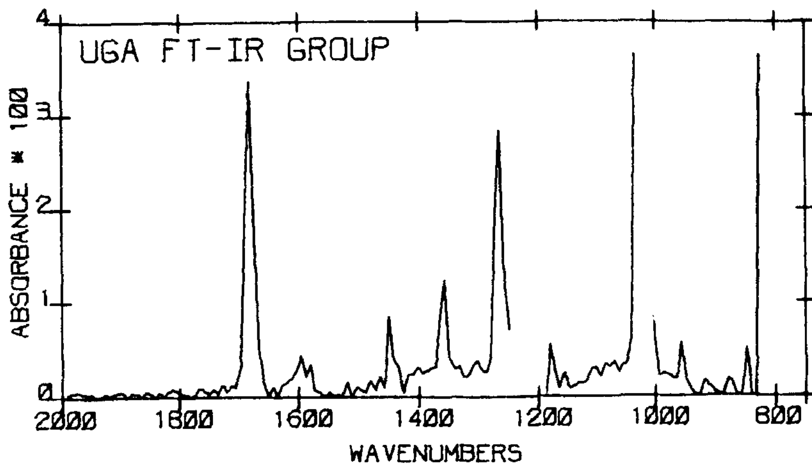
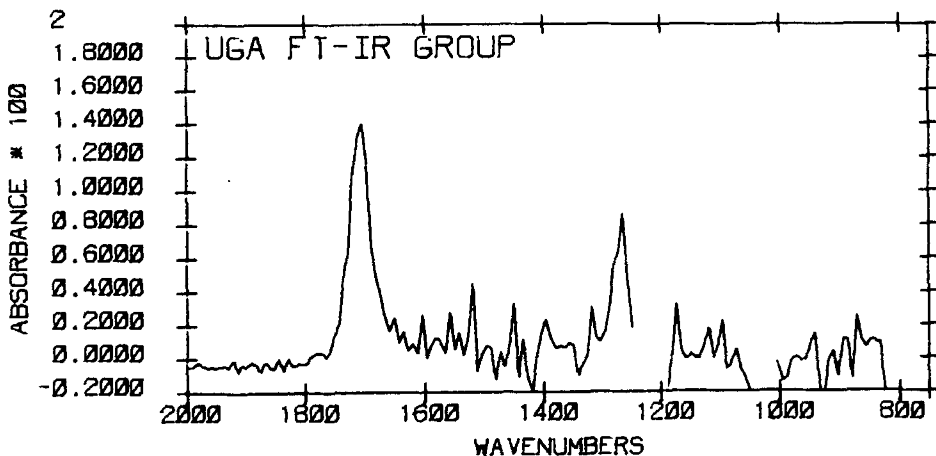
the first separation (3:3:1 chloroform, water, methanol solvent system). These are high SNR spectra, even for *p*-nitrophenol and phenol which elute last and undergo greater diffusion. At the 0.5 mm pathlength it is still possible to decrease the amount of injection. Thus, it is not necessary for each component to be in excess of a milligram for CCC/FT-IR spectrometry to be useful.

To increase the sensitivity of the system the pathlength was increased to 1.0 mm. As expected the 1.0 mm pathlength resulted in numerous areas of solvent opacity and severe loss of spectral information. Due to these problems it was found best to use a pathlength of 0.5 mm in cases where the sensitivity must be maximized.

CCC/FT-IR spectrometry can be implemented for applications where the analyst is not limited by sample size. The high eluent concentrations make complex solvent removal procedures unnecessary, and a much simpler flow cell





4. Spectra of a) 200 μg of acetophenone, b) 270 μg of benzoic acid, c) 230 μg of *p*-nitrophenol, and d) 210 μg of phenol at a pathlength of 0.5 mm.

(continued)

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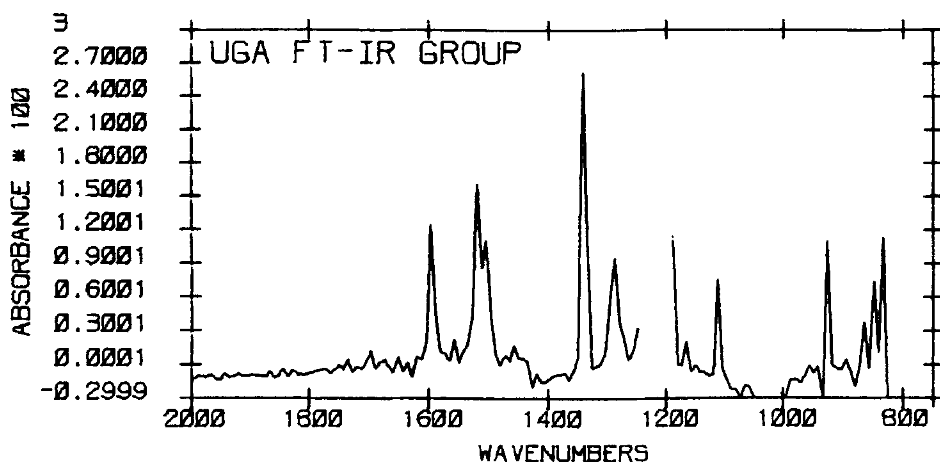

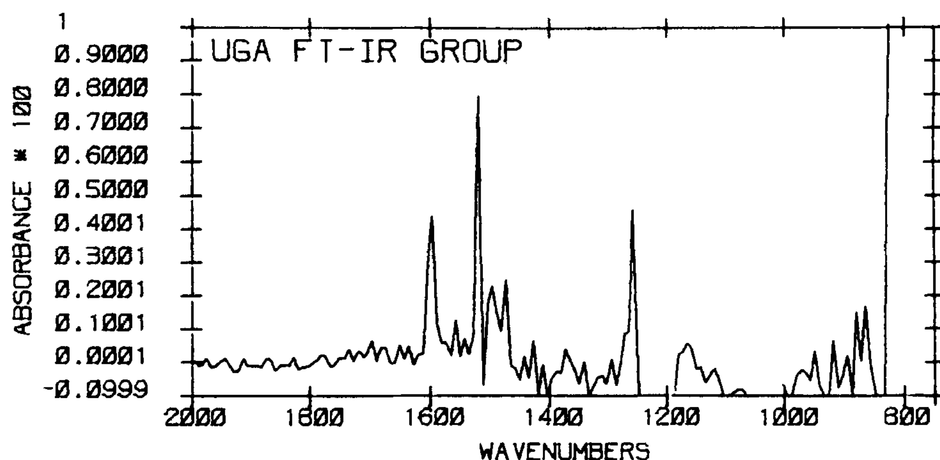

Fig. 4 (continued)

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interface can be used instead. The pathlength of the cell can be adjusted according to the quantity of sample injected. In cases where several milligrams are injected a short pathlength cell (e.g. 0.025 mm or 0.1 mm) could be used in order to minimize solvent opacity problems. Spectra of

compounds present in microgram quantities can be obtained by using a pathlength of 0.5 mm, although solvent opacity problems are augmented at this longer pathlength. Both water and methanol can be used in the separation as part of the stationary phase, without interfering with the eluate spectra. This system should be especially useful to users of countercurrent chromatography to obtain valuable structural information with an FT-IR spectrometer.

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